2021

HHLA2 & IGPR1 roles in tumor progression and metastasis

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

HHLA2 & IGPR1 ROLES IN TUMOR PROGRESSION AND METASTASIS

by

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B.S. Interamerican University of Puerto Rico Ponce, 2017

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2021

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ACKNOWLEDGMENTS

My sincere gratitude to Dr. Nader Rahimi, Dr. Razie Amraei and the whole lab team for an incredible and high-quality research experience. I would also like to thank my advisor Dr. Stephanie Oberhaus for helping through all this journey and supporting me in difficult times. And ultimately and most importantly my family and girlfriend that have always supported me during the way and whom without this would not have been possible. This work was supported in part through grants from the NIH/NCI (R21CA191970, R21CA193958 and CTSI grant 1UL1TR001430 to N.R.).

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ABSTRACT

The metastatic pathways determine the process by which cancer cells give rise to a metastatic lesion in a new tissue or organ. Cell-cell adhesion is a central aspect of many of these metastatic pathways. Cell adhesion molecules belonging to the immunoglobulin superfamily (Ig-SF) commonly play a central role in cell-cell adhesion, and a number of these molecules have been associated with cancer progression and a metastatic phenotype. HERV-H LTR-associating protein 2 (HHLA2) and immunoglobulin-containing and proline-rich receptor-1 (IGPR1) are two recently discovered IG-SF cellular adhesion molecules of the B7 and CD 28 family that are overexpressed in several cancer cell lines and contribute to increased growth, metastatic phenotype and decreased immune cell infiltration status. Regarding the accumulating evidence on the potential interaction between IGPR1 and HHLA2 in immune regulation we sought to explore the effects of this proposed interaction in the phosphorylation of IGPR1 on SER220. Our results show that HHLA2 reduces phosphorylation of IGPR1 at Ser220 in a in vitro co-culture assay. Taken together, our data suggests that IGPR-1/HHLA2 pathway could regulate cell invasion and metastasis by stimulating and increased prosurvival and metastatic phenotype.

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LIST OF ABBREVIATIONS

CAM	Cell Adhesion Molecule
ECM	Extracellular Matrix
HEK-293	Human Embryonic Kidney
HHLA2	HERV–H LTR-associating 2
IgSFCAM	Immunoglobulin Superfamily Cellular adhesion molecules
IGPR-1	Immunoglobulin-containing and proline-rich receptor-1
JAM-A	Junctional Adhesion Molecule-A
MAPK	Mitogen Activated Protein Kinase
SPIN90	SH3 Protein Interacting with Nck90

INTRODUCTION

Cell proliferation, migration, and differentiation are critically important during the development of all organisms, and it is the overall coordination of these activities that leads to the formation of complex structures such as tissues and organs [1]. These cellular processes are modulated by the interaction of cells with each other and with their microenvironment. Cell adhesion molecules (CAMs) facilitate these interactions and are essential during development and for maintenance of structural and functional integrity of the tissue architecture and requires highly dynamic cell-cell and cell-matrix interactions involving different types of surface receptors. Among these receptors, adhesion molecules, cadherins, and integrins play a major role by recognizing and interacting with other cell adhesion receptors on neighboring cells and by binding components of the ECM [54]. Besides providing mechanical anchorage to the cell, these structures also are of functional importance; they transduce signals from the ECM and neighboring cells that are critical for survival and proliferation. Loss of these signals frequently initiates apoptosis [55].[2,3]. CAMs include cadherins, integrins, selectins, and the immunoglobulin superfamily (IgSF). In normal tissue, CAM expression is tightly regulated. However, aberrant expression of CAMs disrupts normal cell-cell and cell-matrix interactions, freeing cells from normal check points and constraints, and facilitating tumor formation and metastasis [5]. One of the most important and ubiquitous types of adhesive interactions required for the maintenance of solid tissues is the cadherin adhesion molecules. Cadherins are transmembrane Ca^{2+}- dependent homophilic adhesion receptors that are well known to play

important roles in cell recognition and cell sorting during development [56]. However, they continue to be expressed at high levels in virtually all solid tissues. There are many members of the classic cadherin family, but E-cadherin in epithelial tissues has been the most studied in the context of stable adhesions. Continued expression and functional activity of E-cadherin are required for cells to remain tightly associated in the epithelium, and in its absence the many other cell adhesion and cell junction proteins expressed in epithelial cells are not capable of supporting intercellular adhesion. In its capacity to maintain the overall state of adhesion between epithelial cells, E-cadherin is thought to act as an important suppressor of epithelial tumor cell invasiveness and metastasis [57]. Cell adhesion to ECM is essentially achieved through integrin-mediated linkage to extracellular ECM molecules and intracellular cytoskeleton. The large extracellular domain of integrins bind to ECM molecules while the intracellular domain is linked to cytoskeleton through intracellular focal adhesions (FAs) [59]. FAs are supramolecular complexes formed by more than 150 different proteins, including kinases, scaffold and adaptor proteins, as well as actin linking proteins, they also mediate intracellular signaling pathways and are dynamic structures which assemble, disperse, and recycle during cell migration [58,60,61]. They also play critical roles in regulating other biological processes, such as apoptosis, proliferation, survival, and differentiation through integrin-mediated down-stream signaling pathways [61]. During cancer differentiation and metastasis processes, up-regulation of integrins has been linked to cancer invasiveness [62-64]. Selectins are vascular cell adhesion molecules involved in adhesive interactions of leukocytes and platelets and endothelium within the blood circulation. There are three members of the selectin family: P-, E-, and L-selectin. The physiological

functions of selectins are well described in processes of inflammation, immune response, wound repair, and hemostasis [65]. P-selectin is present in the storage granules of platelets (α-granules) and endothelial cells (Weibel-Palade bodies), thus enabling rapid translocation on cell surfaces upon activation. Endothelial expression of E-selectin requires *de novo* transcription, leading to expression on activated endothelial cell surfaces several hours after stimulation. L-selectin is constitutively expressed on cell surfaces of almost all leukocyte subpopulations [65,66]. The naturally occurring ligands for the three selectins are mostly mucin-type glycoproteins carrying sialylated, fucosylated glycans [67-70]. In the normal physiological state, epithelial cells line the lumen of hollow organs and are covered by mucins that are either cell surface attached or building soluble layers covering the epithelium. Progression and poor prognosis of carcinomas are associated with enhanced expression of sialylated, fucosylated epithelial mucins. During malignant transformation cell surface glycans undergo dramatic changes that ultimately facilitates disseminating tumor cells in the blood ,not only interaction with endothelial cell selectins and tissue infiltration via rolling adhesion and diapedesis, but also permits malignant cells to bind platelets via selectins and coat themselves from their microenvironment potentiating even more the infiltration and seeding potential via platelet recruitment to endothelial lining ruptures forming microemboli that facilitate their arrest in the vasculature [71-73]

To survive in tissue, epithelial cells must anchor to extracellular matrix (ECM), as detachment from it induces a specific programed cell death known as anoikis [6]. Tumorigenic transformation due to genetic alterations allows tumor cells to survive

and proliferate without the requirement of anchorage to ECM (that is, anchorage-independent growth) [7]. Emerging evidence suggests that as tumor cells lose the requirement for anchorage dependency for growth and survival, they increasingly rely on their ability to adhere to each other (that is, multicellular aggregation) for survival [9,10]. Invasive tumors frequently invade stroma in large groups by the mechanism of collective cell migration [11,12]. Circulating tumors of colorectal, breast, and prostate cancer are often present in aggregates and not in a single cell [13-17]. Tumor cell aggregation also significantly influences the cells' response to cytotoxic drugs, as tumor cells in a spheroid environment are more resistant to radiation and chemotherapeutic agents, a phenomenon originally coined multicellular resistance (MCR) [18-20].

Accumulating evidence on the role of cell–cell adhesion in tumor progression, and response to therapeutics suggests that tumor cell–cell interaction provides tumor cells an adaptive survival mechanism by which they overcome the need for anchorage dependency to ECM and evade the cytotoxic effects of chemotherapeutics. Additionally, this resistance to anoikis plays a major role in tumor metastasis conferring survival to these tumor cells after detachment from their primary location and they can then travel through the circulatory systems and subsequently use this increase in cell adhesion phenotype to anchor themselves in distant tissues [1,26,27]. Cancer metastasis is a process in which cancer cells disseminate from the primary tumor, settle and grow at a site other than the primary tumor site. The metastatic pathway describes the process by which cancer cells give rise to a metastatic lesion in a new tissue or organ. It consists of interconnecting steps all of which must be successfully completed to result in a metastasis. Cell-cell adhesion is a key aspect of many

of these steps. Adhesion molecules belonging to the immunoglobulin superfamily (Ig-SF) commonly play a central role in cell-cell adhesion, and a number of these molecules have been associated with cancer progression and a metastatic phenotype. During metastatic spread, tumor cells disseminate to sites distant from the primary tumor, using cell migration mechanisms that are similar, if not identical, to normal physiological processes. The metastatic process consists of five sequential steps: (1) tumor cell proliferation and angiogenesis; (2) local cell invasion; (3) intravasation and dissemination; (4) extravasation; (5) metastatic colonization and proliferation [21-25]. Tumor cells also must escape immunological attack during any of these stages to survive by employing immune evasion tactics such as the overexpression of immune checkpoints that cause T-Cell exhaustion and dampening of the immunosurveillance and reduced elimination of carcinogenic cells. IgSF members have been implicated in most, if not all, of these processes [28,29].

IgSF Superfamily: Tumorigenesis and Metastasis

The IgSF is one of the largest and most diverse families of proteins in the body. Members of the IgSF include major histocompatibility complex class I and II molecules, proteins of the T cell receptor complex, virus receptors [53] , and cell surface glycoproteins. The definitive characteristic of the IgSF members is the presence of one or more immunoglobulin like domains, which have a characteristic sandwich structure composed of two opposing antiparallel β-pleated sheets, stabilized by a disulphide bridge. Most of the IgSF members are type I transmembrane proteins, which typically consist of an extracellular domain (which contains one or more Ig-like domains), a single transmembrane domain, and a cytoplasmic tail. IgSF members mediate calcium-

independent adhesion through their N-terminal Ig-like domains, which commonly bind other Ig-like domains of the same structure on an opposing cell surface (homophilic adhesion) but may also interact with integrins and carbohydrates (heterophilic adhesion). The C-terminal intracellular domains of IgSF members often interact with cytoskeletal or adaptor proteins. In this way, the extracellular interactions of IgSF CAMs can lead to signaling within the cell, enabling these proteins to function in a wide range of normal biological processes such as their vital role in embryonic development and by modulating cell–cell adhesion and cell migration, as well as pathological events such as inflammation and tumorigenesis. Several IgSF members have been identified as biomarkers for cancer progression. For example, MCAM (also called CD146, Mel-Cam, Muc18, and S-Endo1) has been implicated in the progression of melanoma, as well as in breast and prostate cancer. Similarly, IgSF members such as L1CAM (CD171), NCAM (CD56), PECAM-1 (CD31), ALCAM (CD166), and ICAM-1 (CD54) have been associated with metastatic progression in a range of cancers including melanoma, glioma, breast, ovarian, endometrial, prostate, and colon cancer. IGPR1 and HHLA2 are new members of the IgSF CAM family with potential involvement in the progression and metastatic phenotype [1].

IGPR1:

Immunoglobulin-containing and proline-rich receptor-1 (IGPR-1), a previously uncharacterized protein, was identified as a novel member in Ig-containing family of cell adhesion molecules. Ig containing adhesion molecules are involved in regulation of cell-cell adhesion and cell migration in normal development and pathological conditions like inflammation and cancer. Furthermore, Ig containing adhesion molecules play a role in

specificity of cell-cell recognition while their Ig domain participate in protein-protein and protein-ligand interaction. IGPR-1 is expressed in various cell types including epithelial and endothelial cells. Interestingly, IGPR-1 gene has been found in advanced eukaryotes including primates, bovine, dolphins, canines, horses, etc. whereas, it is not present in rat or mouse genome. IGPR-1 is mainly expressed by cells of epithelial origin, including bronchial epithelial cells of lung, breast glandular and lobular epithelia cells, urothelium of the bladder, skin epidermis, epithelium of gastrointestinal, and rectum. Moreover, endometrial glands of the uterus, the ureter, fallopian tube epithelium, colonic epithelium, small bowl epithelium, stomach epithelium, including both chief and parietal cells, trophoblastic epithelium of placenta, and pancreatic acinar cells were all positive for IGPR-1. IGPR1 is found in a glycosylated form in the thymus, placenta, heart, small intestine, skin, and kidney with a molecular weight of 55 kDa while in the skeletal muscle, brain, colon, lung, and ovary IGPR1 is unglycosylated with an apparent molecular weight of 35 kDa. Beyond epithelial cells that were positive for IGPR-1, endothelial cells present in vein and arteries also consistently were positive for IGPR-1. IGPR1 is expressed in various organs; however, its expression is mainly in epithelial and endothelial cell types. IGPR-1 is comprised of three major domains: extracellular, transmembrane and intracellular. The extracellular domain of IGPR-1 contains a single immunoglobulin domain followed by a single transmembrane domain and a proline-rich intracellular domain (Fig 1).

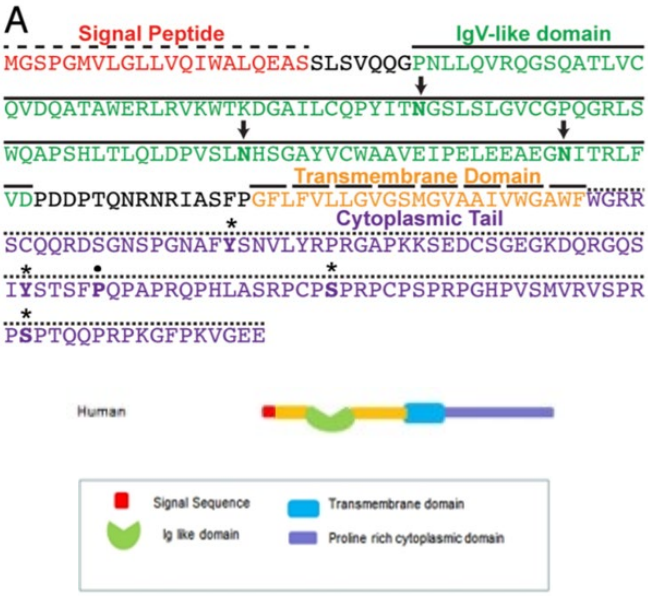

Figure 1. The deduced amino acid sequence of human IGPR-1. Amino acids 1–22 are a putative signal sequence (red). The immunoglobulin domain of IGPR-1 is shown (green), together with a proline-rich cytoplasmic region (purple).

IGPR-1 seems to adapt a typical Ig V-like fold consisting of a sandwich of two antiparallel β-sheets. The immunoglobulin-containing extracellular domain is required for IGPR-1 to mediate endothelial cell–cell interaction and barrier function. IGPR-1 is localized at cell membrane in a di-sulfide linked cis-dimer structure. IGPR1 trans-homophilic dimerization with neighboring cells triggers phosphorylation of serine 220 at cytoplasmic region which is essential for IGPR-1 activation (Fig 2).

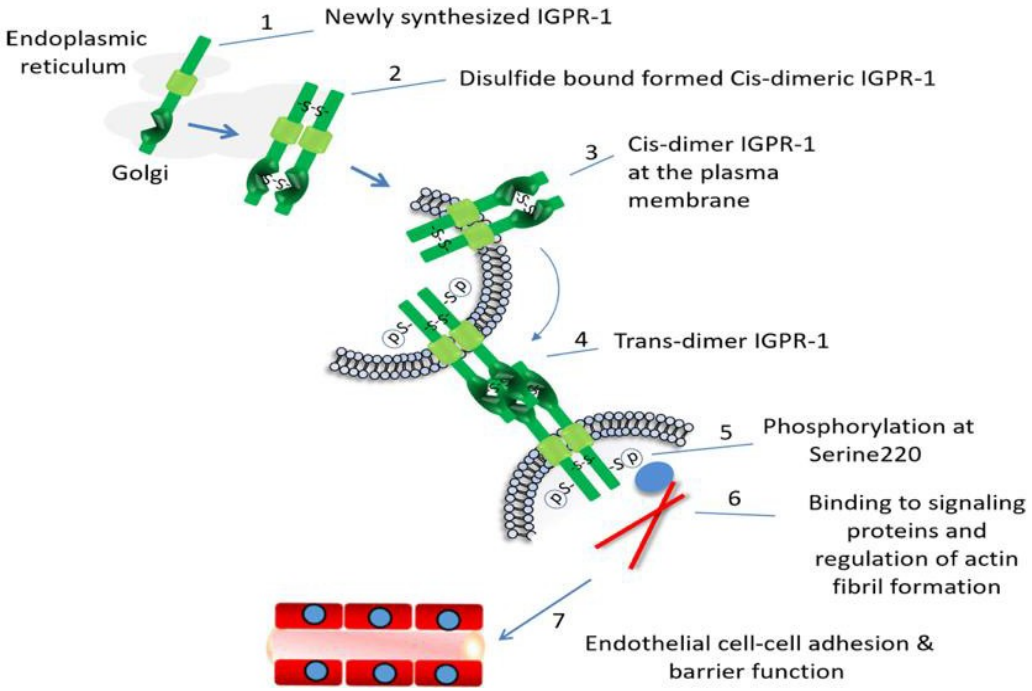

Figure 2. Mechanism of IGPR-1 activation and function. (Wang et al., 2016)

Deletion of the extracellular domain (ΔN-IGPR-1), which eliminated the trans-homophilic dimerization of IGPR-1 abrogated phosphorylation of Ser220 indicating that trans-homophilic dimerization of IGPR-1 regulates Ser220 phosphorylation [30]. Importantly, IGPR-1 is highly glycosylated at its extracellular region and additional analysis showed that using glycosylation inhibitors caused IGPR-1 degradation. In addition, it was demonstrated that IGPR-1 is localized at adherens junctions in endothelial cells where it

regulates cell-cell adhesion and cell migration and it is required for angiogenesis. Further studies have shown that IGPR-1 plays a major role in vascular permeability and endothelial barrier function. To be more precise, IGPR-1 expression in porcine aortic cells (PAE) significantly increased trans-endothelial electrical resistance (TEER). Additionally, using fluorescently labeled dextran to verify vascular permeability, IGPR-1 expression decreased vascular permeability in PAE cells. The proline-rich intracellular domain of IGPR-1 is phosphorylated at multiple serine residues and associates with various Src homology 3 (SH3) domain-containing proteins, including SPIN90/WISH (SH3 protein interacting with Nck), potentially linking IGPR-1 to actin polymerization via N-WASP and Arp2/3 complex [29-30]. IGPR-1 expression is elevated in human primary colon cancers and promotes *in vivo* and *in vitro* tumor growth. Interfering with IGPR-1 activity by shRNA or blocking antibody inhibited growth of HCT116 cells. IGPR-1 distinctively promotes tumor growth by increasing multicellular aggregation of tumor cells. More importantly, IGPR-1 expression in colon tumor cells significantly contributes to the development of resistance to the chemotherapeutic drug, doxorubicin [2]. In addition to its adhesive function, IGPR-1 is proposed to bind to HHLA2, a member of the B7 family of costimulatory molecules involved in the activation and downregulation of T lymphocytes [31,32]. However, the biological relevance of HHLA2 interaction with IGPR1 remains unknown.

HHLA2

HHLA2 (B7H7) has recently been identified as a new member of the B7 family member [33,34]. HHLA2 was initially discovered as a gene in the Immunoglobulin (Ig) superfamily when screening the human genome for human endogenous retroviral (HERV) long

terminal repeat (LTR) sequences which provide polyadenylation signals [35]. Hence the name, HHLA2, is short for HERV-H LTR-associating 2. HHLA2 orthologs appear to be present in a wide range of species such as fish, frog, giant panda, monkey and human, but not in laboratory mouse and rat strains. The HHLA2 protein has amino acid similarity of 23 to 33% to the other human B7 family molecules and phylogenetically it is most similar to B7-H3 and B7x (B7-H4/B7S1). The predicted structure of HHLA2 is a type I transmembrane molecule with three extracellular Ig domains (Fig 3).

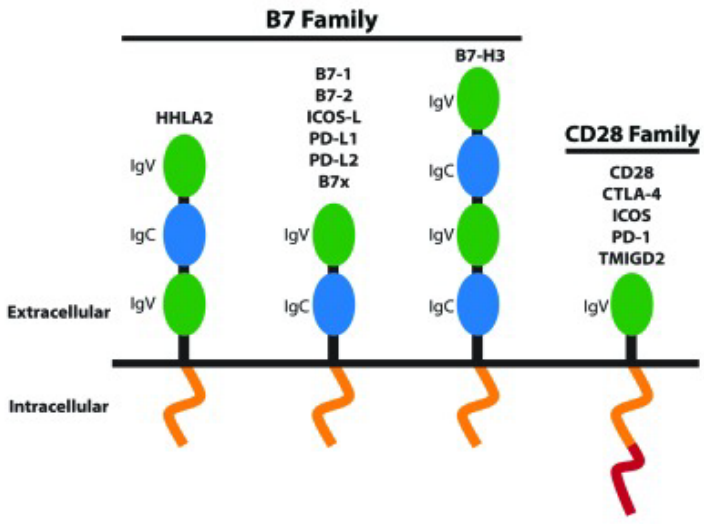

Figure 3. A structural representation of the B7 and CD28 family members.

This is unique as most other B7 family members contain only two Ig domains while human B7-H3 has four Ig domains. Most normal organs do not express HHLA2 at the protein level; however, trophoblastic cells of the placenta and epithelial cells of the gut, kidney, gallbladder and breast expressed this ligand. Additionally, HHLA2 is constitutively expressed on human monocytes and induced on B cells after stimulation with IFN-γ, while primary and secondary lymphoid organs were largely negative, a few scattered cells

appeared to stain positively in these samples. These results reveal that endogenous HHLA2 protein is absent in most normal tissues, but mainly expressed on epithelial cells of a few tissues. HHLA2 does not interact with other known members of the CD28 family or the B7 family but does bind a putative receptor that is constitutively expressed not only on resting and activated T cells but also on APCs. In 2013, Zhu and colleagues found that TMIGD2/IGPR1/CD28 homologue (CD28H) bound to HHLA2 on APCs. In 2015, Janakiram *et al* found that HHLA2-Ig bound to cells expressing Transmembrane and Immunoglobulin Domain Containing 2 (TMIGD2) and TMIGD2-Ig bound strongly to 3T3 cells expressing HHLA2. Since HHLA2 belongs to the B7 family, it is hypothesized that HHLA2 regulates T cell function. HHLA2 immunoglobulin fusion protein (HHLA2-Ig) binds to T cells (resting and activated) and other immune cells demonstrating that there are constitutive receptors on the cell surface. HHLA2-Ig can decrease both CD4 and CD8 T cell proliferation when incubated with anti-CD3. Functionally incubating T cells with HHLA2-Ig decreases the production of several cytokines including IFN-γ, TNF-α, IL-5, IL-10, IL-13, IL-17A, and IL-22. HHLA2 also inhibits IL-2 secretion by T cells in a dose dependent manner. These experiments demonstrate that HHLA2 inhibits T cell proliferation and function. HHLA2 also functions as a costimulatory molecule and increases cytokine production. It is not uncommon for members of the B7 family to have dual functions depending on the immune milieu, receptor engagement or blockade or interaction with different receptors. Overall these studies demonstrate that HHLA2 predominantly functions as a T cell coinhibitory molecule [31-34].

Apoptotic Evasion. The first step in metastasis is the transformation of cells from a normal to a cancerous phenotype. This is when cells acquire characteristics that help them to withstand factors that may limit their metastatic spread. These factors include genotypic stress, tissue hypoxia, nutrient depletion, the accumulation of toxic metabolites, haemodynamic shearing, and loss of adhesion. Most cells encountering these factors will undergo apoptosis (preprogrammed cell death). However, genome expression analysis of metastatic tumors using cDNA microarrays has revealed a strong correlation between tumor progression and the loss of expression of proapoptotic genes, with a concomitant gain in expression of antiapoptotic genes [24,25]. Thus, the acquisition of apoptotic resistance in cells under stress is the first requirement in tumor progression toward metastasis. Classically, genotypic stress due to genomic instability through DNA mutation, chromosomal rearrangement, and epigenetic alteration will trigger apoptosis through the tumor suppressor p53 (TS P53) pathway. In many tumor cells, the expression of TS P53 is lost, enabling them to avoid apoptotic death [36]. However, this accounts for only 40% of cells that undergo malignant transformation. Recent reports have indicated that aberrant expression of CAMs such as the IgSF members provides antiapoptotic signals that may account for the other 60% of malignant transformation. For example, Campodónico et al. reported that the functional blockade of NCAM led to susceptibility to apoptosis in murine lung tumor cells and suggested that NCAM expression may be linked to apoptotic resistance in these cells. This resistance seems to be due to activation of the transcription factor, nuclear factor kappa B (NF-κB), whose downstream targets are antiapoptotic genes

such as B-cell lymphoma/leukemia-x long (Bcl-x1), X-linked inhibitor of apoptosis protein (XIAP), and cellular inhibitor of apoptosis protein (C-IAP) [37-39]. MCAM expression by melanoma cells has also been shown to activate NF-κB via the upstream p38 mitogen-activated protein kinase (MAPK) [40]. Inhibition of MCAM using a blocking monoclonal antibody led to downregulation of p38 MAPK phosphorylation, the suppression of NF-κB activation, and a decrease in tumor growth, possibly due to cell death through apoptosis [41]. Similarly, IGPR-1 increased survival of both HT29 and HCT116 cells in suspension condition and analysis of phosphorylation of p38 in HT29 cells showed that in HT29 cells expressing IGPR-1, phosphorylation of p38 was significantly inhibited, suggesting that the prosurvival effect of IGPR-1 in Colorectal Cancer tumor cells in the absence of adhesion to ECM is mediated by reducing activity of the stress-induced p38. Interfering with IGPR-1 activity by shRNA or blocking antibody inhibited growth of HCT116 cells [2].

Angiogenesis. Acquisition of angiogenesis by tumor cells is considered the most critical step in tumor growth and metastasis. To grow beyond 2 mm in diameter, a tumor needs to undergo angiogenesis, which is often established by hypoxia-induced expression of VEGF and other hypoxia-induced growth factors. It has been shown that IGPR1 stimulates angiogenesis and that modulation of expression of IGPR-1 by ectopic expression or silencing in endothelial cells significantly altered the angiogenic phenotype of endothelial cells in culture and introducing IGPR-1 to tumor cells increased tumor angiogenesis via SPIN90 association through its SH3 domain [29]. Taken together, IGPR-1 promotes multicellular aggregation in tumor cells, increases tumor growth *in vivo* and *in vitro*, and increases the resistance of tumor cells to chemotherapeutic agents.

Dissemination and Immune Evasion Once tumor growth has reached a critical mass, the metastatic spread of tumor cells is dependent on their dissociation from the primary tumor and migration towards the systemic circulation. Primary tumors with invasive properties usually display reduced intercellular adhesion, which allows cells to break away from the parental cell mass. Intravasation of tumor cells is not well understood, but it is generally believed that tumor cells can pass easily into the irregular, highly permeable blood vessels formed during tumor angiogenesis [42]. Once inside the vasculature, less than 0.1% of these circulating tumor cells (CTCs) are estimated to remain viable after 24 hours and less than 0.01% survive to generate metastases [43]. This may be due to anoikis, the result of fluid shear forces, or immunological attack. Anoikis is an apoptotic process triggered by the loss of cell-matrix interactions and the ability to overcome this is crucial for CTC survival [44]. As beforementioned IGPR1 confers anoikis resistance and increased survival in suspension conditions. Moreover, tumor multicellular aggregation promotes cell survival and expression of IGPR-1 by tumor cells contributes to multicellular aggregation and tumor cell survival. Tumor cells often coopt to cellular aggregation by interacting with each other or other cell types to lessen the cytotoxic effects of chemotherapeutics, a phenomenon known as ‘multicellular resistance’. Multicellular resistance occurs in response to a variety of anti-cancer strategies, including chemotherapy and ionizing radiation and IGPR-1 by modulating phosphorylation of histone H2AX acts to reduce the sensitivity of tumor cells toward the DNA-damaging agent, doxorubicin. IGPR-1 provides a unique ability to evade apoptosis, grow without anchoring to ECM, and develop resistance to conventional chemotherapies. In addition to its adhesive function and

prosurvival effects, IGPR-1 binds to HHLA2, a member of the B7 family of coinhibitory molecules involved in the downregulation of T lymphocytes activation [2]. Given that HHLA2 inhibits IGPR1 function it is possible that, through downregulation or inhibition of IGPR1 phosphorylation at Ser220, HHLA2 increases endothelial cell permeability and facilitates tumor cell infiltration to the vasculature by loosening tumor cell cohesion and disrupting endothelial cell junctions [1,22-24,29-33,43]. In accordance with this hypothesis, it has been found that HHLA2 expression in Osteosarcoma, PD-L1 negative non-small cell lung carcinoma, bladder urothelial carcinoma, colorectal carcinoma and triple negative breast cancer increases metastatic phenotype, lymph node metastasis and tumor aggressiveness and is correlated with a decreased T-cell infiltration status [31,32, 45-51]. In agreement with a possible role of IGPR-1 in cell adhesion and cell–cell interaction, expression of IGPR-1 in PAE cells and B16F cells inhibits cell migration [29]. Of interest, the reduced migration of these cells by IGPR-1 also correlates with the inhibition of phosphorylation of paxillin at tyrosine 118 (Y118). Phosphorylation of Y118 of paxillin is linked to inhibition of cell migration suggesting that IGPR-1 inhibits cellular migration, in part by stimulating dephosphorylation of paxillin either by increasing protein phosphatase activity or preventing its phosphorylation by inhibiting tyrosine kinases such as Src family kinases and focal adhesion kinases, which are involved in the phosphorylation of paxillin [29]. The wide expression of HHLA2 in human cancers and its association with more invasive disease suggests that HHLA2 potentially plays an important role in tumor evolution and metastases through immune suppression, inhibition of IGPR1

mediated tumor cell adhesiveness, increased cellular migration phenotype and the disruption of endothelial cell permeability.

Specific aims:

The overall goal of this project was to investigate the potential effect of HHLA2 in IGPR-1 activation. Specifically, the specific aims of this project were:

Specific aim I: Examine expression of profile of IGPR-1 and HHLA2 in a panel of human tumor cell lines.

Specific aim II: Test the hypothesis that HHLA2 inhibits activation of IGPR-1.

METHODS

Antibodies and Reagents

Rabbit polyclonal antibody against HHLA2 protein was purchased from Fisher Scientific (Rockford, IL). It was used diluted 1:1000 in Blotto and used in Western blots to detect the presence of HHLA2 in HEK-293 cells. Rabbit polyclonal anti-IGPR-1 antibody was made against a peptide derived from the cytoplasmic domain of IGPR-1 as previously described [29]. Anti-IGPR1 antibody was used diluted 1:5000 in Blotto solution and used for Western blot analysis to detect expression of IGPR1 in HEK-293 cells and colon cancer cell lines. Anti-phospho-serine 220 IGPR-1 (pSer220) antibody was developed using a peptide containing phospho-serine 220 and further purified by peptide affinity chromatography using phospho-serine220 containing peptide as previously described [30]. Anti-pSer220 antibody was used in 1:1000 dilutions for Western blot analysis.

Cell Culture

Human Embryonic Kidney cells (HEK-293)(ATCC) were grown and maintained in Dulbecco's Modified Eagle's Media (DMEM) containing 10% fetal bovine serum (FBS) and 50 units/mL penicillin and streptomycin. Cells were placed in a humidified incubator at 37°C and 5% CO_2. Cells were sub-cultured by using 2 mL of 0.05% trypsin/EDTA for 2 minutes in 47 mm plates while at 80-90% confluency and subcultured in a 2:10 dilutions. Colon cancer cell line

lysates were provided by the Dr. Rahimi Lab from previous experiments and were stored in -20 Cº freezer.

Plasmids and constructs

The cDNA of HHLA2-HisTag was purchased from Invitrogen (Carlsbad, CA), was PCR amplified and cloned into retroviral vector pMSCV with puromycin resistance gene (Invitrogen, Carlsbad, CA) via XhoI and EcoRI. Similarly, cDNA corresponding to IGPR1 was purchased from Invitrogen (Carlsbad, CA), and was PCR amplified and cloned into retroviral vector pMSCV via HindIII and SalI restriction sites. To confirm for insertion the vectors where analyzed by agarose gel electrophoresis. Expression was confirmed in subsequent transfection steps via Western Blot analysis (see below).

Cell Transfection

HEK-293 cells were grown in DMEM 10% FBS and 50 units/mL penicillin and streptomycin medium to 60% confluency .Prior to transfection, the medium was removed and 2 mL of serum free DMEM was added to each 47 mm cell culture dish. DNA-PEI (polyethylenimine) mixtures were prepared containing 400µl of serum-free DMEM, 9µg of PEI and 3µg DNA from pMSCV-HHLA2-HisTag or pMSCV-IGPR1 and incubated for 15 minutes at room temperature. The HHLA2 DNA-PEI, IGPR1 DNA-PEI and PEI mixtures were added separately

to each plate respectively (3 plates) and the cells were returned to 37°C incubator. After 6 hours, 2mL of DMEM 10% FBS was added to each plate and the next day (16-20 hours), all of the medium was replaced with 4 ml DMEM 10% FBS (2 µg/ml puromycin) for selection of transfected colonies.

Retrovirus Production and Transduction

In order to perform retroviral virus production, 293-derived retroviral packaging cell line (293GPG) cells were maintained at 90% confluence in 293 GPG Growth Media containing Dulbecco's modified eagle medium (DMEM), 10% (vol/vol) fetal bovine serum (FBS) , 2 mM L-glutamine ,50 units/ml each of penicillin and streptomycin , 1 ,ug/ml tetracycline, 2 µg/ml puromycin and 0.3 mg/ml G418 [52]. Then the 293 GPG cells were transfected with pMSCV-HHLA2-HisTag or pMSCV-IGPR1. After 24 hours, the media was replaced with viral producing medium (293 GPG media without tetracycline) and at 72, 96, 120, and 144 hours the virus was collected as described [52]. . , 4ml of virus and 2µl of polybrene were added to HEK-293 cells at 70% confluence in a 60mm plate. DMEM 10% FBS with appropriate selection antibiotic (2 µg/ml puromycin and 0.3 mg/ml G418) was added to select for transduced cells. Cells were monitored for 3 days in the incubator at 37 ºC to ensure survival in antibiotic media before confirming successful transduction. After successful transduction cells were expanded for further analysis.

Western Blot Analysis

HEK-293 , HEK-293 HHLA2-His Tag and HEK-293 IGPR1 cells 60mmculture dishes were placed on ice, rinsed twice with chilled H/S buffer [20 mM Hepes (pH 7.4) and 150 mM NaCl] then lysed using lysis buffer containing 10uL/ml Na3VO4 and 15 ul/ml PIC (Protease Inhibitor Cocktail) [500 μM AEBSF 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 150 nM aprotinin, bovine lung, crystalline, 1 μM E-64 protease inhibitor, 0.5 mM EDTA, disodium, and 1 μM leupeptin, hemisulfate] for every mL of EB buffer [10mM Tris- HCl (pH 7.5), 10 mM EDTA, 50 mM NaCl, 50 mM NaF, 1%Triton X-100]. Whole cell lysates were centrifuged. Then, 5X sample buffer [3.8% Tris-base, 50% glycerol, 5% sodium dodecyl sulphate (SDS), 5% β-mercaptoethanol, and .0025% bromophenol blue] was added to each collected supernatant and samples were boiled at 95°C for 5 minutes. Cell lysates were loaded on a 12% SDS polyacrylamide gel and subjected to electrophoresis (SDS-PAGE), then transferred onto polyvinylidene difluoride (PVDF) membranes pre-activated by methanol. PVDF membranes were blocked with Blotto (2% non-fat dry milk and 0.05% Tween-20 in Western Rinse) and for HHLA2 blots we used Bovine Serum Albumin blocking buffer for 1 hour on a rocker at room temperature. Following washing with Western rinse [25 mM Tris -HCl (pH 7.2), 0.05% Tween-20 and 0.15 mM NaCl for 5 minutes at r, membranes were immunoblotted with the respective primary

antibodies for 1 hour at room temperature. After three washes with Western rinse (each time 10 minutes), membranes were incubated with the respective secondary antibodies for 1 hour at room temperature. Following three 10 minutes washes with Western rinse membranes were (0.1M Tris pH 8.5, 0.2mM coumaric acid and 1.25mM Luminol mixed with 0.12% (w/w) hydrogen peroxide in a 1:1 ratio)

RESULTS

Expression of HHLA2 and IGPR-1 in human cancer cell lines

Expression of IGPR1 and HHLA2 is shown for several Renal and Colon cancer cell lines (Fig. 4). There seems to be a discrepancy between the relative expressions of both which raises the question for future research as to how HHLA2 expression levels affect IGPR1 expression in a co-expression cell line system and eventually in an wild type cancer cell line.

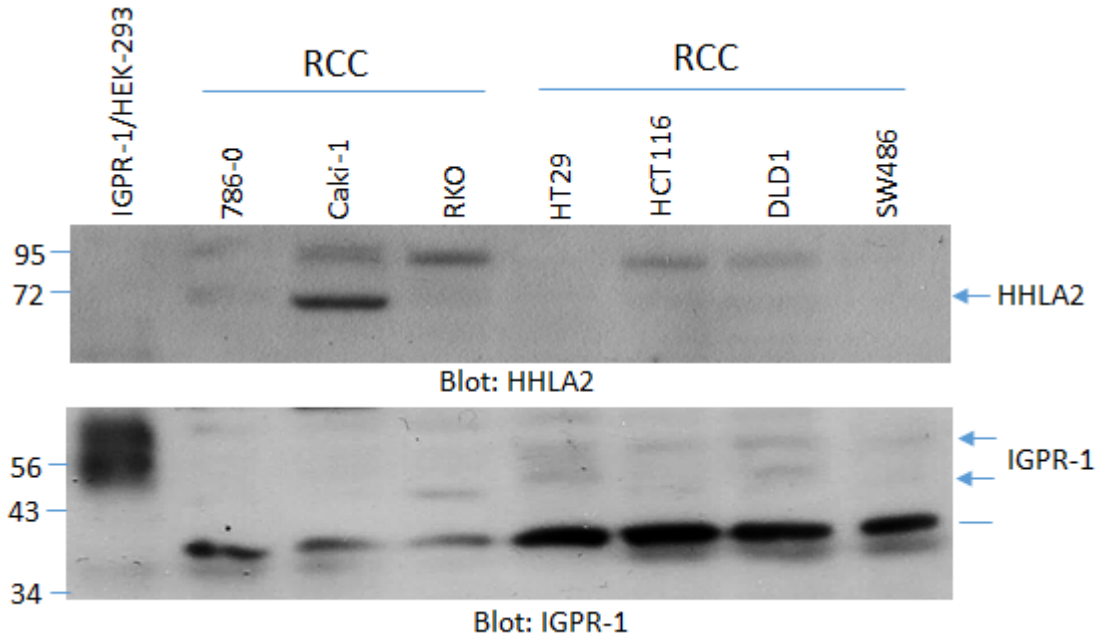

Figure 4. HHLA2 and IGPR1 expression in renal and colon cancer cell lines.

HHLA2 inhibits phosphorylation of IGPR1 at pSer220

Considering the important role of phosphorylation of IGPR1 at Ser220 we sought to explore the potential function of HHLA2 in the phosphorylation of IGPR1 at Ser220. The result showed that co-culture of HEK293 HHLA2 and IGPR1 for 48 hours significantly reduced phosphorylation of IGPR1 at Ser220 (Fig.2)

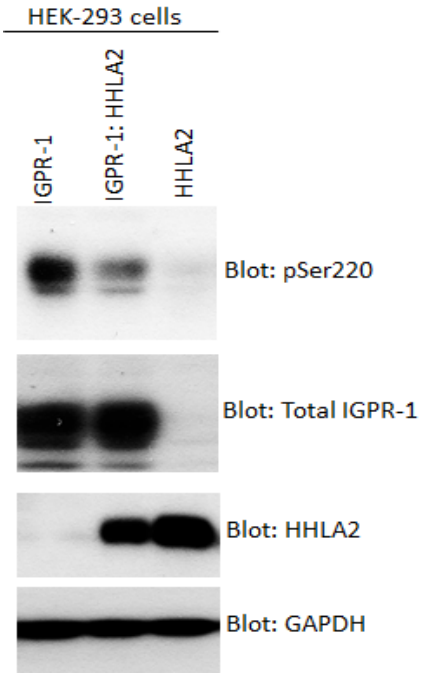

Fig. 5 IGPR1 phosphorylation at Ser220 is inhibited after co-culture with HHLA2 overexpressing cell line.

DISCUSSION

The metastatic pathway describes the process by which cancer cells give rise to a metastatic lesion in a new tissue or organ. It consists of interconnecting steps all of which must be successfully completed to result in a metastasis. Cell-cell adhesion is a key aspect of many of these steps. Adhesion molecules belonging to the immunoglobulin superfamily (Ig-SF) commonly play a central role in cell-cell adhesion, and a number of these molecules have been associated with cancer progression and a metastatic phenotype. The metastatic process consists of five sequential steps: (1) tumor cell proliferation and angiogenesis; (2) local cell invasion; (3) intravasation and dissemination; (4) extravasation; (5) metastatic colonization and proliferation [21-25]. Tumor cells also must escape immunological attack during any of these stages to survive by employing immune evasion tactics such as the overexpression of immune checkpoints that cause T-Cell exhaustion and dampening of the immunosurveillance and reduced elimination of carcinogenic cells.

IGPR-1 is a novel adhesion molecule, which is widely expressed in endothelial and epithelial cells [29-30]. IGPR1 trans-homophilic dimerization with neighboring cells triggers phosphorylation of serine 220 at cytoplasmic region which is essential for IGPR-1 activation and function in cell-cell adhesion, endothelial barrier function, and regulation of angiogenesis which contribute to increased growth in the primary tumor and stimulation of angiogenesis [29-30]. Expression of IGPR-1 in PAE cells and B16F cells inhibits cell migration and inhibition of IGPR1 increases vascular permeability [29]. IGPR-1 expression is elevated in human primary colon cancers and promotes *in vivo* and *in vitro* tumor growth. Interfering with IGPR-1 activity by shRNA or blocking antibody

inhibited growth of HCT116 cells, suggesting that targeting IGPR-1 could offer a novel anti-cancer strategy. IGPR-1 distinctively promotes tumor growth by increasing multicellular aggregation of tumor cells. In addition to its adhesive function and prosurvival effects, IGPR-1 binds to HHLA2, a member of the B7 family of coinhibitory molecules involved in the downregulation of T lymphocytes activation [2].

HHLA2 is a newly discovered T cell immune checkpoint molecule that belongs to the B7 family of ligands. It predominantly functions to inhibit T cell proliferation and T cell cytokine responses. HHLA2 is expressed on few normal tissues but it is expressed in various human cancers. High expression of HHLA2 in human cancer of lung, breast, and osteosarcoma is associated with worse prognostic features and increased lymph node positivity. It has been found that HHLA2 expression in Osteosarcoma, PD-L1 negative non-small cell lung carcinoma, bladder urothelial carcinoma, colorectal carcinoma and triple negative breast cancer increases metastatic phenotype, lymph node metastasis and tumor aggressiveness and is correlated with a decreased T-cell infiltration status [31,32, 45-51].

Considering that HHLA2, inhibits phosphorylation of pSer220 on IGPR1 it is possible that this interaction may contribute to local cell invasion in the primary tumor site by disrupting cell cohesion in the epithelia. Additionally, this inhibition may potentiate intravasation into the vasculature by interfering with endothelial cell-cell adhesiveness and increasing vascular permeability through IGPR inhibition. Moreover, HHLA2 may potentiate dissemination throughout the vasculature and through the epithelial layer by increasing cellular migration phenotype of tumor cells by inhibition of IGPR1. Ultimately, inhibition

of CD4 and CD8 through HHLA2 T-Cell inhibition may potentiate even further the metastatic phenotype by shielding the traveling multicellular aggregates from immune surveillance and attack [33].

Given the contradictory roles of IGPR1 in promoting survival and growth and HHLA2 negatively regulating IGPR1 phosphorylation at pSer220 it is imperative to explore the roles of these seemingly antagonistic proteins in the potentiation of growth and metastatic phenotype. Additionally, it is important to explore the timing and microenvironmental conditions that stimulate the expression of these two proteins as well as localization of these proteins relative to the primary tumor mass. Probably IGPR1 expression comes first to allow primary tumor cells to override growth signaling pathways through inhibition of p38MAPK and protect themselves from possible chemotherapeutic agents by increasing the number of focal adhesions through IGPR1 trans-dimerization between epithelial tumor cells. In addition, IGPR also helps to establish blood flow to the tumor by stimulating angiogenesis [2,29]. Then HHLA2 expression by peripheral tumor cells of the primary tumor may help them increase vascular permeability, detach from other IGPR1 expressing outside of the mass and evade immune elimination by downregulating T-Cell Activation. The wide expression of HHLA2 in human cancers and its association with more invasive disease suggests that HHLA2 potentially plays an important role in tumor evolution and metastases through immune suppression, inhibition of IGPR1 mediated tumor cell adhesiveness, increased cellular migration phenotype and the disruption of endothelial cell permeability. Hopefully, further studies on this inhibitory HHLA2 pathway may lead to new therapies for human cancers and possibly autoimmune diseases.

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